

Summary of Telephonic interview

At the outset, Applicants wish to thank Examiners Hama and Paras for taking the time on February 2, 2007, to discuss the rejections issued on November 30, 2006. In that paper, Examiner Hama maintained the rejections of claims 1-12 and 39 under 35 U.S.C. §§101 and 112, first paragraph. As the undersigned explained during the interview, these rejections are unwarranted since AHSP deficiency does correlate with human disease. During the interview, the uses of the claimed transgenic mice were discussed and it was agreed that AHSP transgenic mice of the invention correlate with diseases involving defective hemoglobin assembly and ineffective erythropoiesis, e.g., β -thalassemia. Additionally, Examiner Paras agreed that the use of the AHSP knock-out mice of the invention to produce anti-AHSP antibodies would also be a credible, specific, and substantial utility. AHSP is a highly conserved protein and utilization of the knock out mice should result in the generation of a panel of antibodies with diverse epitope specificity as the protein would not be seen as a "self antigen" by the mouse immune system.

In accordance with the Examiners' helpful suggestions, the following remarks are provided which emphasize and underscore the arguments presented in Applicant's previous response. Specifically, as exemplified in the specification, the AHSP gene is involved in specific disease states, and accordingly, AHSP KO mice have utility for production of AHSP antibodies, studying the diseases associated with AHSP insufficiency, and screening agents which correct for such deficiencies. Additionally, the Examiner is requested to consider a research article

describing this work which is attached hereto. See Kong et al. J. of Clinical Investigation 114:1457-1466 (2004).

This Statement of the Substance of Interview is being filed with the response to the November 30, 2006 Official Action containing the Interview Summary Form, as required by the same.

REMARKS

The November 30, 2006, Official Action has been carefully reviewed. In light of the following amendments and remarks, favorable reconsideration and allowance of the application are respectfully requested.

At the outset, it is noted that a shortened statutory response period of three (3) months was set forth in the November 30, 2006, Official Action. Therefore, the initial due date for response is February 28, 2007. This response is being filed within the initial 3 month response period.

The Examiner has maintained the rejection of claims 1-12 and 39 under 35 U.S.C. §101 because the claimed invention allegedly lacks a patentable utility. As a result, the Examiner also maintained the rejection of claims 1-12 and 39 under 35 U.S.C. §112, first paragraph contending that since the invention allegedly lacks utility, one of skill in the art would not know how to use the claimed invention.

The foregoing rejections constitute all of the grounds set forth in the November 30, 2006, Official Action for refusing the present application. In view of the reasons set forth in this response, Applicants respectfully submit that the rejections under 35 U.S.C. §§101 and 112, first paragraph of claims 1-12 and 39 as set forth in the November 30, 2006, Official Action cannot be maintained.

CLAIMS 1-12 AND 39 SATISFY THE UTILITY REQUIREMENTS OF 35

U.S.C. §101

The Examiner has maintained the rejection of claims 1-12 and 39 under 35 U.S.C. §101 for allegedly lacking either a credible, specific, or substantial utility. It is the Examiner's position that none of the utilities asserted by Applicants in the August 28, 2006, response to the May 23, 2006, Official Action meet the standards for §101 since AHSP allegedly does not have any role in human disease, and, therefore, the mice of the invention cannot be used in screens to identify therapeutically effective agents.

Applicants respectfully disagree with the Examiner's position as indicated during the telephonic interview. The MPEP states at §2107.02(III) (A) that "an applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. §101." Applicants respectfully submit that the Examiner has failed to overcome this presumption.

Claim 1 describes the phenotype of the claimed transgenic animals of the invention. Clearly, these AHSP knock-out animals could be used to identify and screen therapeutic agents which alter the phenotypes associated with AHSP insufficiency. These agents would be useful to restore normal erythrocyte function and could be used to treat human diseases like β -thalassemia which involve excess hemoglobin production characterized by red blood cell damage and generation of reactive oxygen species which damage cellular proteins. β -thalassemia is a type of anemia that involves deficiencies with red blood cells during erythropoiesis. Indeed, page 43, lines 29 through page 44, line 17 highlight the defects in hemoglobin assembly that underlies the pathophysiology of ineffective

erythropoiesis in β -thalassemia which is characterized by high concentrations of α -hemoglobin that form toxic aggregates. For example, at page 48, lines 16 through 31, the generation of an AHSP knock-out mouse in which erythrocytes contained denatured hemoglobin inclusions and exhibited defective hemoglobin metabolism is described. Moreover, at page 14, line 31 through page 15, line 17, AHSP is identified as a protein that, among other things, protects free α -hemoglobin from precipitation which is involved in the formation of α -inclusion bodies in Alzheimer's disease. Further discussion of the AHSP knock-out mouse in relation to β -thalassemia can be found at page 63, lines 9-25. The mice of the invention clearly recapitulate key features of the pathology observed in β -thalassemia, and as indicated on page 31, starting at line 12, the mice of the invention can be used to identify agents which alter AHSP deficiency in anemia.

In the response dated August 28, 2006, Applicants also noted an additional utility in Example V on pages 69, line 28 through page 73. Clearly, as discussed above, AHSP knock out mice of the invention can be utilized to produce AHSP antibodies exhibiting a wider array of epitope specificity. Accordingly, Applicants take exception to the Examiner's assertion that this feature is not germane to the utility of the instantly claimed knock out mice. Such antibodies have utility for intracellular localization, epitope mapping, and immunoprecipitation studies for characterizing proteins that associate intracellularly with AHSP as discussed in Example V.

Furthermore, at page 65, line 19 through page 66, line 15, the inventors disclose that altered AHSP activity is implicated in other human disease such as hemolytic anemia,

and glucose-6-phosphate dehydrogenase deficiency involved in malaria. Additionally, the involvement of aberrant AHSP function in transmissible spongiform encephalopathy (or prion disease) is discussed in the paragraph starting on page 31, line 27 of the specification.

Clearly, AHSP deficiency has a role in human disease, and the instantly claimed transgenic animals are supported by the above credible, specific and substantial utilities. Once an invention has been determined to be "useful for some purpose, it becomes unnecessary to decide whether it is in fact useful for the other purposes 'indicated' in the specification as possibly useful." MPEP §2107.02 (I). Applicants respectfully submit that, contrary to the Examiner's position, a skilled artisan would find the utilities above, in particular the use of the transgenic mice to screen therapeutic agents which alter phenotypes associated with AHSP deficiency, to be a specific, substantial, and well-established utility.

In light of the above-described utilities, Applicants submit that nothing more is needed to satisfy the requirements of 35 U.S.C. §101. Accordingly, Applicants respectfully submit that the rejection of claims 1-12 and 39 is untenable and respectfully request its withdrawal.

**THE SUBJECT MATTER OF CLAIMS 1-12 AND 39 IS FULLY ENABLED
BY THE DISCLOSURE IN THE SPECIFICATION AS FILED**

The Examiner has maintained the rejection of claims 1-12 and 39 for failing to satisfy the enablement requirement of 35 U.S.C. §112, first paragraph. Specifically, the Examiner asserts that the claims contain subject matter which was not described in the specification in such a way

as to enable one skilled in the art to which it pertains to make or use the invention. It is the Examiner's position that the specification does not teach whether the mice of the invention are a model for any AHSP-associated disease or disorder, and that the phenotype of the knock-out animals is allegedly unpredictable. As such, the Examiner contends that the use of the claimed mice is not readily apparent, and, therefore, the invention is not enabled.

Applicants respectfully disagree with the Examiner's position. As stated in the MPEP at §2107.02(I), "an applicant need only make one credible assertion of specific utility for the claimed invention to satisfy 35 U.S.C. §101 and 35 U.S.C. §112." As demonstrated in the preceding section, there is at least one clear, substantial and well-established utility for the instantly claimed transgenic mice and the methods of use thereof. As a consequence, the requirements of 35 U.S.C. §112, first paragraph have been satisfied.

Regarding the Examiner's rejection of claims 1-12 and 39 under 35 U.S.C. §112, first paragraph, the instant specification clearly enables practice of the invention encompassed by the claims. As described hereinabove the specification teaches that, among other diseases, AHSP deficiency exacerbates β -thalassemia, and associated defects in hemoglobin assembly. This anemic disorder is clinically characterized by erythrocytes having abnormal spiculated morphology, reduced lifespan, increased production of reactive oxygen species (ROS), and precipitated hemoglobin.

The Examiner has applied the enablement factors provided at §2164.01(a) of the MPEP and concluded that, in view of the allegedly undeveloped and unpredictable state

of the art and the alleged absence of guidance with regard to transgenic animals, a skilled artisan would have to perform undue experimentation to practice the full scope of the claimed invention.

Applicants respectfully disagree with the Examiner's position. In In re Wands, 8 USPQ2d 1400 (1988), the Federal Circuit Court of Appeals held that engaging in experimentation to practice a claimed invention does not render the disclosure non-enabling as long as the experimentation required is not "undue." The Court stated that: "The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness ... The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." In re Wands, 8 USPQ2d 1400, 1404 (1988).

Applicants contend that the instant specification discloses the preferred methods to generate the transgenic mice presently claimed. Exemplary methods of transgenic animal production are disclosed in the specification (see, Example II starting at page 46 of the specification). Applicants submit that a skilled artisan could effectively apply these methods to produce the transgenic animals presently claimed, and that any experimentation required for generating the claimed transgenic animals is standard in the art and cannot be considered undue. In fact, Applicants generated the claimed transgenic mouse of the invention as indicated at page 48 of the specification, and

the reduction to practice was fairly straightforward and in no way required "undue experimentation." Applicants contend that the state of the art of transgenic animals does not reach the extreme unpredictability asserted by the Examiner. Furthermore, Applicants have demonstrated that erythrocytes obtained from the AHSP knock-out mice have abnormal spiculated morphology, reduced lifespan, increased production of reactive oxygen species, and precipitated hemoglobin. In view of the successful generation of the transgenic mice of the invention with the desired phenotype, it should be apparent that the invention is both fully enabled by the disclosure and that a skilled artisan would know how to use the mouse based on the specification.

Applicants also note on page 8 of the instant Official Action, the Examiner states that "Applicant indicated that the test compounds or agents are screened for their ability to substitute for AHSP and restore AHSP activity" but indicates that the claims are not read as such. In the interest of expediting prosecution, Applicants have amended claim 9 to recite screening for agents which alter AHSP deficiency-related phenotypes. Support for this amendment can be found in the specification at page 31, line 13 and in claim 1. Applicants are screening for agents that affect AHSP deficiency in the knock-out mouse of claim 1 as recited in step a) of claim 9. In regard to this reason for rejection, a skilled artisan is clearly enabled to generate the claimed AHSP knock-out mouse by following the teaching of Example II, and then can administer a test compound to said mouse and assess the mouse for phenotypic alterations as recited in claims 9-11 and 39.

In light of the present claim amendments, Applicants respectfully submit that the skilled artisan would be

readily able to practice the invention encompassed by these claims without undue experimentation since the instant specification "provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." In re Wands, 8 USPQ2d 1400, 1404 (1988). Accordingly, the rejection of the claims under 35 U.S.C. §112, first paragraph is untenable and should be withdrawn.

CONCLUSION

It is respectfully requested that the amendments presented herewith be entered in this application. These amendments and remarks, which are based on the discussion between the Examiners and the undersigned during the telephonic interview, are believed to clearly place the pending claims in condition for allowance. Therefore, it is respectfully urged that the rejections set forth in the November 30, 2006, Official Action be withdrawn and that this application be passed to issue.

In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to telephone the undersigned at the phone number give below.

Respectfully submitted,

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Enclosure: J. of Clin. Investigation 114:1457-1466 (2004)



Loss of α -hemoglobin–stabilizing protein impairs erythropoiesis and exacerbates β -thalassemia

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Hemoglobin (Hb) A production during red blood cell development is coordinated to minimize the deleterious effects of free α - and β -Hb subunits, which are unstable and cytotoxic. The α -Hb–stabilizing protein (AHSP) is an erythroid protein that specifically binds α -Hb and prevents its precipitation *in vitro*, which suggests that it may function to limit free α -Hb toxicities *in vivo*. We investigated this possibility through gene ablation and biochemical studies. *AHSP*^{−/−} erythrocytes contained hemoglobin precipitates and were short-lived. In hematopoietic tissues, erythroid precursors were elevated in number but exhibited increased apoptosis. Consistent with unstable α -Hb, *AHSP*^{−/−} erythrocytes contained increased ROS and evidence of oxidative damage. Moreover, purified recombinant AHSP inhibited ROS production by α -Hb in solution. Finally, loss of AHSP worsened the phenotype of β -thalassemia, a common inherited anemia characterized by excess free α -Hb. Together, the data support a model in which AHSP binds α -Hb transiently to stabilize its conformation and render it biochemically inert prior to Hb A assembly. This function is essential for normal erythropoiesis and, to a greater extent, in β -thalassemia. Our findings raise the possibility that altered AHSP expression levels could modulate the severity of β -thalassemia in humans.

Introduction

Late-stage erythroid development is largely dedicated to the production of the oxygen carrier hemoglobin (Hb) A, a tetramer consisting of two pairs of α -globin and β -globin protein subunits with each monomer bound to a heme moiety. Hb A synthesis is exquisitely coordinated to minimize the accumulation of free α - or β -Hb subunits, which are cytotoxic. Excessive α -Hb is particularly damaging, as evidenced by β -thalassemia, a common inherited anemia in which mutations in the β -globin gene impair the production of β -Hb with consequent buildup of the unpaired α -subunit (1–5). Intact monomeric α -Hb generates ROS that damage cellular proteins, lipids, and nucleic acids (6). In addition, α -Hb is structurally unstable, with a tendency to denature upon oxidation, filling the cytoplasm and cell membrane with precipitated α -globin polypeptides, free heme, porphyrins, and iron, which further propagate ROS production (reviewed in ref. 7). Together, these effects reduce the lifespan of circulating erythrocytes and also impair the viability of erythroid precursors in hematopoietic tissues, causing ineffective erythropoiesis.

Most cells contain compensatory mechanisms to cope with unstable proteins (8). These include molecular chaperones that

stabilize proteins and in some cases facilitate their folding into native structures. In addition, there are degradation pathways that recognize and eliminate improperly folded polypeptides. Accordingly, tissues typically tolerate some protein instability, with disease ensuing only when the compensatory mechanisms become overwhelmed. Several findings indicate that mechanisms to neutralize free α -Hb exist in erythroid cells. First, erythroid precursors contain a small pool of excess free α -Hb with no apparent ill effects (9, 10). In addition, erythropoiesis is typically relatively normal in humans lacking one functional β -globin gene (β -thalassemia trait). Finally, there is frequent unexplained phenotypic diversity among individuals with the same β -thalassemia genotype (reviewed in ref. 11). The last observation could be explained by genetic variations in processes that stabilize or eliminate free α -Hb. Mechanisms to degrade excess free α -Hb in thalassemic erythroid cells were first recognized by Bank and O'Donnell in 1969 (12) and were later shown to be mediated through ubiquitin-dependent proteolytic pathways by Shaeffer and colleagues (13–15). More recently, we identified α -Hb–stabilizing protein (AHSP), also known as erythroid-associated factor (ERAF), a candidate molecular chaperone for α -Hb (16, 17).

AHSP was identified as an erythroid-specific protein whose gene was induced by the essential transcription factor GATA-1 (16, 17). AHSP heterodimerizes with α -Hb (K_d , approximately 100 nM), but does not bind β -Hb or Hb A. Moreover, α -Hb bound to AHSP is more resistant to oxidant-induced precipitation than α -Hb alone. Based on these findings, we hypothesized that AHSP might protect erythroid cells from α -Hb toxicity by maintaining α -Hb in a stable state prior to its incorporation into Hb A. To test this, we generated *AHSP*^{−/−} mice by gene targeting. Preliminary analysis of these animals revealed abnormal erythrocyte morphology with

Nonstandard abbreviations used: AHSP, α -Hb–stabilizing protein; BFUe, burst-forming unit erythroid; CFUe, CFU erythroid; DCFH, 2',7'-dichlorofluorescein; DNPH, 2,4-dinitrophenylhydrazine; Epo, erythropoietin; Hb, hemoglobin; HDW, Hb distribution width; MCH, mean corpuscular Hb; MCV, mean corpuscular volume; NHS, *N*-hydroxysuccinimide; RDW, rbc distribution width; SBTI, soybean trypsin inhibitor; SCF, stem cell factor; TAU, Triton-acetic acid-urea; TMPD, tetramethyl-*p*-phenylenediamine.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Table 1
Erythrocyte indices of *AHSP*^{+/−} mice

<i>AHSP</i> genotype	+/+ (<i>n</i> = 8)	−/− (<i>n</i> = 9)	<i>P</i> value
Hb	16.9 ± 0.6	14.5 ± 1.0	< 0.001
Hct	55.8 ± 2.1	48.7 ± 3.2	< 0.001
Reticulocytes (%)	2.2 ± 1.0	4.4 ± 1.8	0.19
MCV	53.5 ± 1.6	45.6 ± 1.2	< 0.001
MCH	16.2 ± 0.5	13.6 ± 0.3	< 0.001
RDW	13.1 ± 2.2	19.1 ± 2.0	< 0.001
HDW	1.5 ± 0.1	2.3 ± 0.1	< 0.001

Hb, g/dl; hematocrit (Hct), %; MCV, fl; MCH, pg; RDW, %; HDW, g/dl. Values shown are mean ± standard deviation; *n* = number of mice analyzed. Hematological indices from *AHSP* heterozygotes did not differ significantly from those of wild-type mice.

hemoglobin precipitates (Heinz bodies) (17). Here we have examined these mutant mice in greater detail to gain further insights into the molecular actions of AHSP *in vivo*. We found that loss of AHSP reduced the lifespan of circulating red blood cells and also caused increased apoptosis of erythroid precursors. These effects were mediated, at least in part, by increased production of ROS with consequent damage to Hb A and other cellular components. Moreover, AHSP blocked ROS production by α -Hb directly. Finally, through interbreeding of mutant mice, we show that loss of AHSP worsened the severity of β -thalassemia. Together, these findings indicate that AHSP acts as protein-specific molecular chaperone that detoxifies free α -Hb during normal erythropoiesis and in pathological states of α -Hb excess.

Results

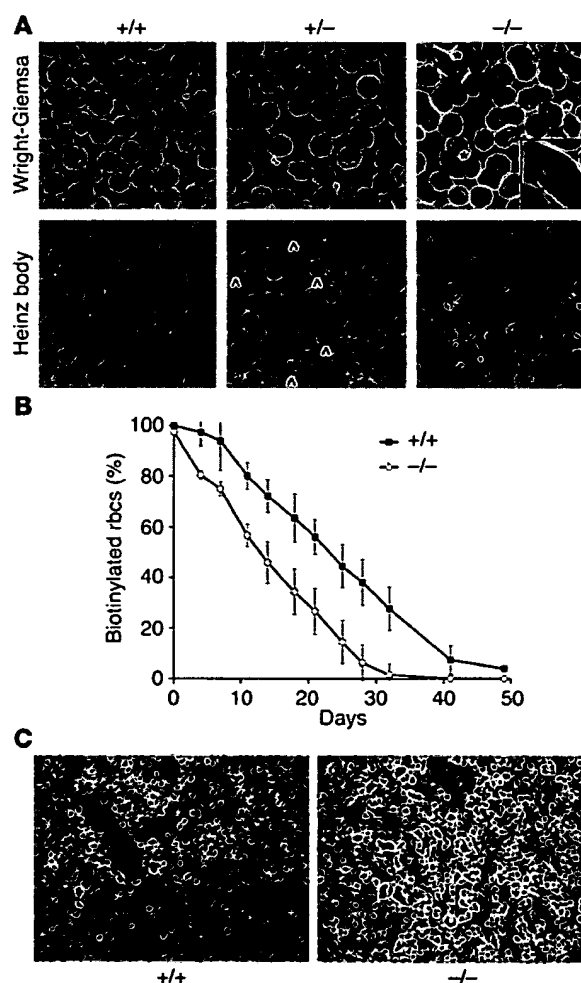
Loss of AHSP causes hemolytic anemia with globin chain precipitation. To study the hematopoietic consequences of AHSP loss, we disrupted the gene in mice as described previously (17). Our targeting strategy replaced the entire protein-encoding region with a *phosphoglycerate kinase promoter–neomycin-resistance* gene (*PGK-Neo*^R) cassette flanked by *loxP* recombination sites. The hematopoietic abnormalities in *AHSP*^{−/−} animals reported below were identical when the *PGK-Neo*^R expression cassette was either present or removed by Cre-mediated recombination (not shown).

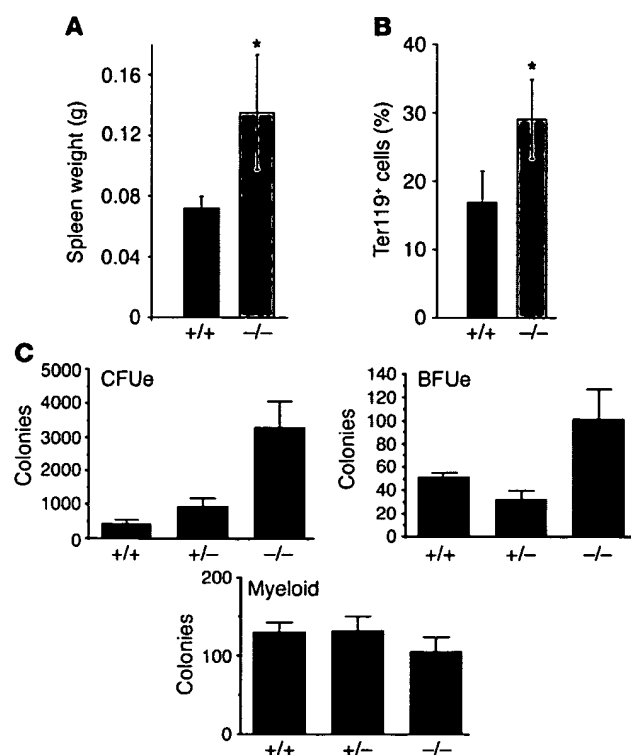
Figure 1

AHSP^{+/−} erythrocytes exhibit abnormal morphology, hemoglobin precipitates (Heinz bodies), and reduced lifespan. (A) Wright-Giemsa staining (upper panels) shows eosinophilic inclusion bodies (*) in *AHSP*^{+/−} erythrocytes. Heinz body staining, which detects denatured globin chains (lower panels), is weakly positive in some *AHSP*^{+/−} erythrocytes (indicated by carets) and is strongly positive in *AHSP*^{−/−} cells. The boxed area in the top right panel shows an enlargement of an inclusion body. Original magnification, ×1,000. (B) Erythrocyte survival kinetics determined by biotin labeling. Circulating erythrocytes in 5 animals of each genotype were biotinylated at days −2 and −1. Beginning at day 0, approximately 5 μ l of blood was removed from the tail vein at the indicated time points, and the fraction of biotin-labeled erythrocytes was quantified by flow cytometry. The half-life of wild-type red blood cells was 22 days, whereas that of the *AHSP*^{+/−} red blood cells was 12 days. *AHSP*^{−/−} erythrocytes exhibited normal survival kinetics (not shown). (C) Prussian blue staining for cellular iron in spleen. Increased iron in the *AHSP*^{+/−} spleen reflects accelerated clearance of erythroid cells by the reticuloendothelial system. Original magnification, ×200.

AHSP^{+/−} and *AHSP*^{−/−} mice were born at the expected mendelian ratios and displayed no gross abnormalities compared with wild-type (*AHSP*^{+/+}) littermates. *AHSP*^{+/−} mice exhibited normal lifespans up to at least 18 months of age. As expected, there was no AHSP RNA or AHSP protein detected in hematopoietic tissues of *AHSP*^{−/−} animals (17). Hematological analysis of the gene-targeted mice performed using an automated analyzer (Bayer ADVIA 120) revealed several new findings not appreciated previously (Table 1). No abnormalities in platelets or white blood cells were detected, but there were several obvious erythroid defects. *AHSP*^{+/−} animals exhibited a mild but significant anemia associated with small red blood cells (low mean corpuscular volume [MCV]) containing decreased Hb (low mean corpuscular Hb [MCH]). There was significant variation in the size and hemoglobin content of the mutant erythrocytes, as evidenced by increased rbc distribution width (RDW) and Hb distribution width (HDW), respectively. The reticulocyte count was elevated in a subset of *AHSP*^{+/−} mice, indicating increased erythrocyte production to compensate for accelerated loss or destruction.

The blood smears of *AHSP*^{+/−} mice showed numerous morphologic abnormalities, including irregular size and shape, target cells, and spiculated cells (Figure 1A, upper right panel). There were increased polychromatophilic cells representing newly synthesized erythrocytes; many of these contained eosinophilic inclusions. Inclusion bodies were also apparent in *AHSP*^{+/−} erythrocytes after staining with



**Figure 2**

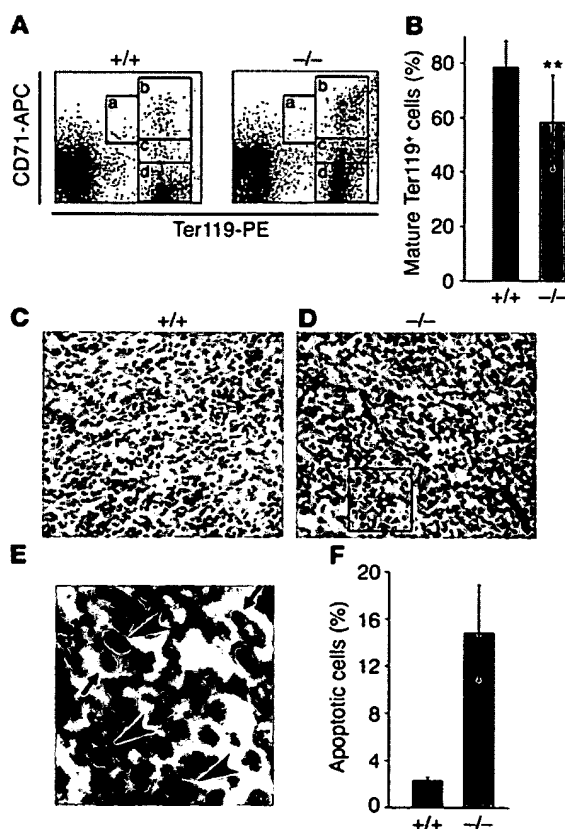
Erythroid hyperplasia in *AHSP*^{-/-} mice. (A) Increased spleen weight in *AHSP*^{-/-} mice. (B) Elevated proportion of Ter119⁺ erythroid cells in *AHSP*^{-/-} mice, as determined by flow cytometry. (C) Methylcellulose progenitor assays. **P* < 0.005. *n* = 6 for each genotype.

was then monitored over a 50-day period by removal of small samples (approximately 5 μ l) of blood from the tail vein, staining with FITC-streptavidin, and quantification of the fraction of labeled cells using flow cytometry. Erythrocytes from normal littermates exhibited a half-life of about 22 days, in accordance with previous studies (18). In contrast, the half-life of *AHSP*^{-/-} erythrocytes was significantly shortened to about 12 days. Hence, loss of AHSP causes significant premature destruction of circulating red blood cells. Histological examination of spleens from *AHSP*^{-/-} mice showed engulfment of erythroid cells by macrophage (erythrophagocytosis; not shown) and increased macrophage iron, as detected by Prussian blue staining (Figure 1C). These findings are consistent with accelerated removal of mutant erythrocytes and/or erythroid precursors by the reticuloendothelial system (also see below).

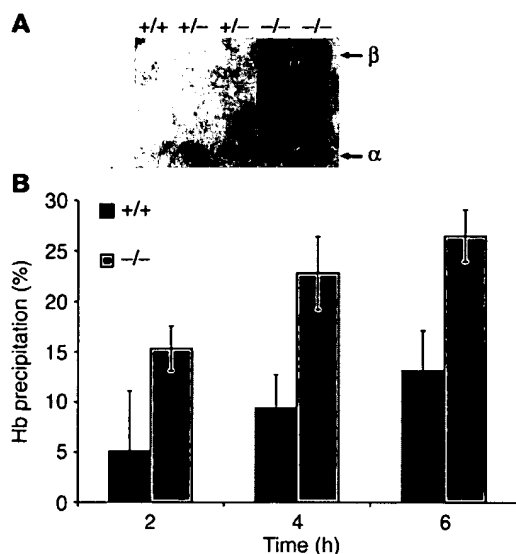
Hyperplasia and excessive apoptosis of *AHSP*^{-/-} erythroid precursors. In most disorders of erythrocyte destruction, there is a compensatory increase in production of erythroid precursors in hematopoietic tissues. The spleen, a major site of erythropoiesis in mice, was significantly enlarged in *AHSP*^{-/-} mice (Figure 2A). Flow cytometry showed an increased proportion of erythroid precursors in *AHSP*^{-/-} spleens, as measured by expression of the late-stage erythroid-specific cell surface marker Ter119 (Figure 2B). To further examine

crystal violet, which detects denatured globin chains (Heinz bodies, Figure 1A, lower panels). Of note, *AHSP*^{-/-} erythrocytes also contained occasional Heinz bodies, suggesting haploinsufficiency effects (see below). Unstable denatured Hbs can accelerate erythrocyte destruction by causing intravascular hemolysis and/or sequestration by the reticuloendothelial system. In support of this, the presence of large inclusions only in polychromatophilic cells suggests that these are rapidly cleared from the circulation. This preferential loss of nascent erythroid cells could result in a reticulocyte count that is lower than expected for the degree of erythrocyte destruction.

To determine the effects of AHSP loss on erythrocyte lifespan more directly, we injected animals with *N*-hydroxysuccinimide-biotin (NHS-biotin), which labeled nearly all circulating red blood cells with biotin (Figure 1B). The survival of tagged erythrocytes

**Figure 3**

Ineffective erythropoiesis in *AHSP*^{-/-} mice. (A) Analysis of splenic erythroid precursors according to levels of Ter119 and CD71 expression. Regions a through d (boxed areas) represent increasingly mature stages of erythroid development (19). APC, allophycocyanin. PE, phycoerythrin. (B) Ratio of mature to immature erythroid precursors in spleen calculated according to levels of Ter119 and CD71 expression defined by flow cytometry gates in panel A: Percent mature cells = number of cells in d / (a + b + c + d); *n* = 6 animals for each genotype; ***P* < 0.05. (C–E) Detection of apoptosis in erythroid precursors. Spleen sections were stained for the erythroid surface marker Ter119 (brown) and for nuclear endonucleolytic cleavage using the TUNEL assay (red). Examples of normal viable erythroid precursors (Ter119⁺TUNEL⁻) are indicated by arrows. Examples of apoptotic erythroid precursors (Ter119⁺TUNEL⁺) are indicated by arrowheads. AHSP genotypes are indicated. E is an enlargement of the boxed region in D. Original magnification in C–E, $\times 400$. (F) Percent apoptotic erythroid precursors in *AHSP*^{-/-} mice and wild-type littermates (*n* = 4 mice of each genotype).



erythroid precursors, we disaggregated spleens into single-cell suspensions and seeded them into semisolid medium. This assay assesses the developmental potential of individual cells and identifies 2 types of committed erythroid precursors: burst-forming unit erythroid (BFUe) cells represent early-stage precursors that give rise to large hemoglobinized colonies in the presence of erythropoietin (Epo) and stem cell factor (SCF); CFU erythroid (CFUe) precursors represent a later stage of development and give rise to smaller colonies in the presence of Epo alone. As shown in Figure 2C, the proportions of both BFUe and CFUe precursors were elevated in *AHSP*^{-/-} mice. In contrast, myeloid progenitors, which give rise to granulocyte- and macrophage-containing colonies in appropriate cytokines, were unchanged. Finally, histological analysis showed notable expansion of the splenic red pulp in the knockout animals (not shown). Therefore, splenic enlargement observed in *AHSP*^{-/-} mice is due to expansion of the erythroid compartment. We also detected significant erythroid hyperplasia in the bone marrow of *AHSP*^{-/-} mice, although to a lesser extent than observed in the spleen (not shown). These results reflect increased erythropoietic drive to compensate for accelerated destruction of mature *AHSP*^{-/-} erythrocytes.

In β -thalassemia, accumulation of excess free α -Hb not only damages mature erythrocytes but also induces apoptosis of erythroid precursors in hematopoietic tissues in a process called ineffective

Figure 4

Unstable hemoglobins in *AHSP*^{-/-} erythrocytes. (A) TAU gel analysis of membrane-associated globin chains with the *AHSP* genotypes indicated at the top. Each lane represents membrane skeletons prepared from the same number of erythrocytes. Right margin: α , α -globin; β , β -globin. (B) Isopropanol hemoglobin stability test. Fresh hemolysates were incubated with isopropanol (17% volume/volume) at 37°C, and hemoglobin precipitation was quantified at the indicated times. *AHSP* genotypes are indicated. The hemoglobin stabilities were significantly different at all time points for the two genotypes ($n = 5$ animals of each genotype; $P < 0.01$).

erythropoiesis (1). To investigate whether loss of AHSP causes ineffective erythropoiesis, we examined splenocytes for coexpression of Ter119 and the transferrin receptor CD71, which allows subdivision of erythroid precursors according to their maturation stage (Figure 3A) (19). *AHSP*^{-/-} mice exhibited an elevated proportion of immature erythroid precursors (Ter119 high, CD71 high) compared with that of mature ones (Ter119 high, CD71 low), suggesting that the transition from immature to mature precursor was partially blocked (Figure 3, A and B). This effect could reflect ineffective erythropoiesis, which is characterized by premature death of erythroid precursors in hematopoietic tissues. We assessed this by direct immunohistochemical examination (Figure 3, C–F). Erythroid cells were identified by expression of Ter119 (brown staining in Figure 3, C and D). Apoptotic cells were identified by TUNEL staining, which detects endonucleolytic cleavage of nuclear DNA (red staining in Figure 3, C–E). *AHSP*^{-/-} spleens contained an increased proportion of Ter119-staining cells, consistent with the flow cytometry data in Figure 2B, above. In addition, apoptosis of erythroid precursors

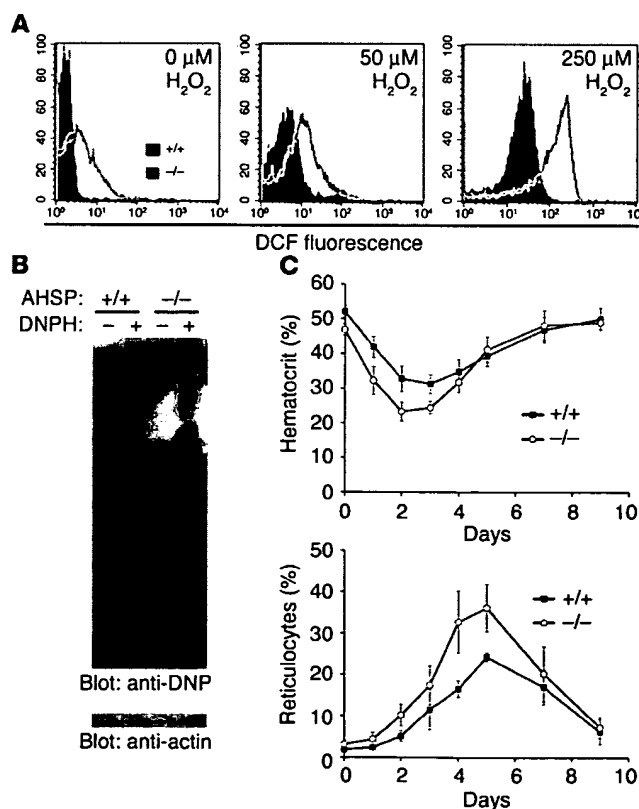
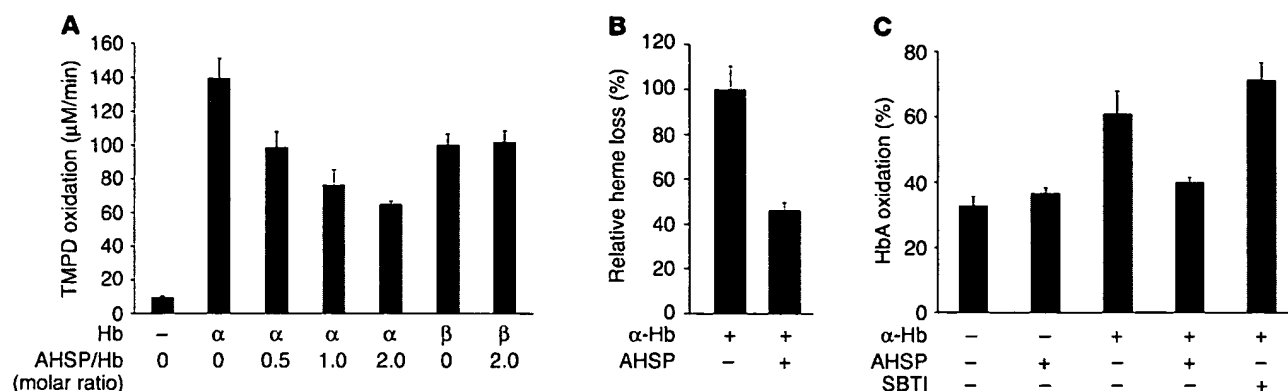


Figure 5

Oxidative stress in *AHSP*^{-/-} erythrocytes. (A) Relative ROS levels in erythrocytes at baseline and with added H₂O₂. ROS were measured by incubation of cells with DCFH, which is converted by ROS to the fluorescent product 2',7'-dichlorofluorescein (DCF). (B) Protein oxidation in erythrocyte lysates. Twenty micrograms of hemolysate was treated with DNP for derivatization of carbonyl groups (by products of protein oxidation). Protein-associated DNP was detected by Western blotting. An identical blot probed with anti-actin (bottom) indicates equal protein loading in each lane. (C) Susceptibility to phenylhydrazine-induced hemolytic anemia. Drug was administered on days -1 and 0. Blood was first sampled on day 0, then hematocrit (top panel) and reticulocyte counts (bottom panel) were assessed daily until recovery. No abnormalities were detected in *AHSP*^{-/-} mice in the experiments described in panels A–C (not shown).

**Figure 6**

AHSP prevents α -Hb-mediated production of ROS and oxidation of Hb A in solution. (A) α -Hb or β -Hb was preincubated with AHSP at the indicated molar ratios for 30 minutes at 4°C, then was mixed with H_2O_2 and TMPD, a ROS indicator dye. The rate of TMPD oxidation, shown on the y axis, was measured by spectrophotometry. Graph shows the average value of three experiments. (B) Effect of AHSP on H_2O_2 -induced heme loss from α -Hb in the experiment in A, as measured by reduced absorbance at 412 nm. The molar ratio of α -Hb to AHSP was 1:1 in the sample on the right. (C) Effect of AHSP or control protein SBTI on α -Hb-mediated oxidation of Hb A. Oxidation of Hb A after 30 minutes at 37°C was measured by spectrophotometry. Where indicated, α -Hb was added to Hb A at a molar ratio of 1:8. AHSP or SBTI was preincubated with α -Hb (1:1 molar ratio) for 30 minutes at 4°C before it was added to Hb A.

was significantly elevated in the mutant animals, as evidenced by an increased proportion of Ter119⁺ cells that were also TUNEL⁺ (Figure 3F). Hence, loss of AHSP not only damages mature erythrocytes, as demonstrated above, but also is toxic to erythroid precursors, similar to the effects of excess α -Hb in β -thalassemia and consistent with a role for AHSP in stabilizing free α -Hb.

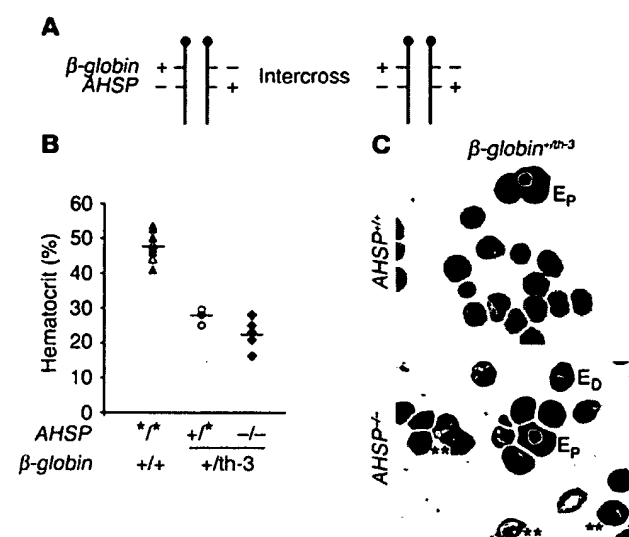
Loss of AHSP results in both α - and β -globin precipitates. Next, we characterized the globin precipitates in *AHSP*^{-/-} mice. In disorders associated with unstable hemoglobins, including thalassemias, denatured globins precipitate onto the cell membrane and damage associated lipids and proteins (20–27). We isolated plasma membranes from erythrocytes of mutant mice and wild-type littermates, extracted them with detergent to remove lipids, and analyzed the resultant membrane skeletons for associated globins by Triton-acetic acid-urea (TAU) gel electrophoresis (Figure 4A). Although we never observed erythrocyte membrane-associated globin chains in control littermates, about half of the *AHSP*^{-/-} (heterozygous) mice examined showed low-level α -globin precipitate, consistent with the presence of occasional Heinz bodies noted in Figure 1A, above. These findings indicate that AHSP haploinsufficiency destabilizes α -Hb to some extent but that this does not exceed a threshold level required for clinically significant erythroid pathology.

Surprisingly, *AHSP*^{-/-} erythrocytes contained both α - and β -globin precipitates at roughly equimolar ratios, despite our previous findings that AHSP specifically binds and stabilizes α -Hb. One explanation for this apparent paradox is that excess free α -Hb can produce ROS that oxidize and destabilize Hb A (28–30), eventually causing it to precipitate. In this case, unstable Hb A should be present in *AHSP*^{-/-} erythrocytes. To test this, we prepared soluble Hb A from fresh hemolysates and examined its stability after the addition of 17% isopropanol, which causes preferential precipitation of unstable hemoglobins (31). As illustrated in Figure 4B, soluble Hb A from *AHSP*^{-/-} erythrocytes precipitated more readily in isopropanol, suggesting prior damage. Taken together with our prior biochemical data, these findings indicate that loss of AHSP destabilizes α -Hb, which then leads to oxidation and eventual precipitation of Hb A.

Oxidative stress in *AHSP*^{-/-} erythrocytes. It is possible that many of the abnormalities observed in *AHSP*^{-/-} erythroid cells are caused by ROS generated from destabilized α -Hb, a potent oxidant. We investigated this by incubating erythroid cells with 2',7'-dichlorofluorescein (DCFH), a ROS indicator that is converted to a fluorescent product upon oxidation. As predicted, mutant erythrocytes contained increased ROS both at baseline and after challenge with H_2O_2 , a physiological ROS precursor (Figure 5A). One consequence of oxidative damage to proteins is the introduction of carbonyl groups onto amino acid side chains. This can be examined by derivatization of protein-associated carbonyl moieties with 2,4-dinitrophenylhydrazine (DNPH), followed by Western blot analysis (32). Using this approach, we found that *AHSP*^{-/-} erythrocytes exhibited distinct oxidative damage, as reflected by elevated levels of carbonyl groups on numerous proteins (Figure 5B).

Erythrocytes with intrinsically elevated oxidative stress exhibit baseline damage and depletion of endogenous antioxidant defenses and therefore are expected to be more sensitive to injury from external oxidants. To test this in *AHSP*^{-/-} mice, we challenged them with phenylhydrazine, an oxidant that causes hemolytic anemia. After treatment with this drug, *AHSP*^{-/-} mice exhibited a greater fall in hematocrit compared to wild-type littermates (Figure 5C, upper panel). Hence, loss of AHSP renders erythrocytes more susceptible to oxidative damage. Of note, the mutant animals responded appropriately to acute anemia by inducing a rapid reticulocyte response (Figure 5C, lower panel). Hence, the partial block to erythroid development in *AHSP*^{-/-} mice described in Figure 3, above, is not severe enough to impair physiologic responses to moderate acute anemia.

AHSP inhibits ROS production by α -Hb through direct mechanisms. Our prior biochemical studies demonstrated that AHSP binds and stabilizes α -Hb specifically (16, 17). Free α -Hb produces ROS by several known mechanisms, and the resultant oxidative damage to erythroid cells is believed to be a major determinant of pathophysiology in states of α -Hb excess, most importantly β -thalassemia (33). AHSP could limit damage from α -Hb by directly inhibiting its ability to produce ROS.

**Figure 7**

Intercrosses of β -globin^{+/th-3} AHSP^{+/-} double-heterozygous mice. The th-3 mutant β -globin allele represents a targeted deletion of the *b1* and *b2* adult globin genes; heterozygous animals exhibit β -thalassemia intermedia, while the homozygous state is lethal in utero. (A) Mating strategy. The β -globin and AHSP genes are physically linked on mouse chromosome 7. Double-heterozygous mice used were the F1 progeny of intercrosses of simple AHSP^{+/-} heterozygotes and β -globin^{+/th-3} heterozygotes, ensuring that the two mutant alleles were on separate chromosomes (*trans* configuration). The genotype frequencies of live-born offspring resulting from F1 intercrosses are described in Table 2. +, wild-type allele; -, deleted allele. (B) Hematocrits of β -globin-AHSP compound mutant embryos, with genotypes shown below the x axis. For comparison, embryos are grouped according to the presence or absence of thalassemia (β -globin^{+/th-3} genotype); thalassemic embryos are subdivided into AHSP-null versus AHSP-wild-type or -heterozygous states. Asterisk denotes either + or - alleles for AHSP. Each symbol represents one embryo analyzed: triangles, β -globin^{+/+} embryos; circles, β -globin^{+/th-3} with at least one wild-type AHSP allele; diamonds, β -globin^{+/th-3} AHSP^{-/-}. Color coding is used to specify the AHSP genotype within each group: black, +/+; white, +/-; red, -/-. (C) Blood smears of β -globin^{+/th-3} embryos with +/+ or -/- AHSP genotypes. Most of the erythrocytes are anucleate definitive (fetal liver derived). Nucleated cells: E_p primitive (yolk sac derived); E₀, definitive. Among thalassemic embryos, those lacking AHSP exhibited more prominent eosinophilic erythrocyte inclusions (examples marked by double asterisks) and increased circulating E₀ cells. Original magnification, $\times 100$.

Heme-containing proteins, including purified α - or β -Hb subunits, catalyze the production of ROS from H₂O₂, in a process that can be quantified using tetramethyl-*p*-phenylenediamine (TMPD), a ROS-sensitive dye that absorbs light at 610 nm upon oxidation (34) (Figure 6A). To assess the effects of AHSP on this process, we preincubated heme proteins with recombinant purified AHSP for 30 minutes prior to the addition of H₂O₂ and TMPD. AHSP, which binds α -Hb at a stoichiometry of 1:1 (16), inhibited ROS production by α -Hb in a dose-dependent fashion. These effects were specific, as AHSP did not inhibit formation of ROS from β -Hb or myoglobin (Figure 6A and not shown). Hence, one mechanism through which AHSP detoxifies α -Hb is to suppress its pro-oxidant activities through direct protein interaction. These findings provide further evidence that elevated ROS in AHSP^{-/-} mice are caused by failure

to stabilize free α -Hb rather than by undefined secondary effects independent of AHSP- α -Hb complex formation.

Oxidation of the heme iron of α -Hb to the ferric form allows it to participate in further redox reactions that can result in protein destabilization and release of the heme moiety. This is reflected by reduced absorbance in the Soret peak at 412 nm (not shown). Preincubation of α -Hb with AHSP inhibited H₂O₂-induced loss of heme, indicating that protein destabilization associated with the oxidative process was reduced (Figure 6B). This result is in accordance with AHSP-mediated suppression of ROS production by α -Hb (Figure 6A, above) and with our previous findings that AHSP protects α -Hb from oxidant-induced precipitation (17).

It is possible that relatively low levels of unstable α -Hb in AHSP^{-/-} erythrocytes propagate ROS production with consequent oxidation and precipitation of Hb A and other cellular components. This would explain the presence of β -globin precipitates and unstable Hb A in mutant erythrocytes (Figure 4, above). To test this, we examined the ability of free α -Hb to promote Hb A oxidation in solution (Figure 6C). Conversion of Hb A to the oxidized Fe(III) form is accompanied by characteristic absorbance changes in the visible spectrum that we monitored via deconvolution to known extinction coefficient spectra. After 30 minutes of incubation at 37°C, about 35% of oxygenated Hb A was converted to the met form; this was not affected by addition of AHSP (Figure 6C). The addition of substoichiometric free α -Hb (0.125 molar ratio) roughly doubled the amount of Hb A oxidation. Hence, free α -Hb can promote Hb A oxidation, presumably through production of ROS. These findings are consistent with those of Scott et al., who showed that introducing free α -Hb into normal erythrocytes promotes Hb A oxidation (29, 30). Preincubation with AHSP strongly inhibited the ability of free α -Hb to induce oxidation of Hb A. In contrast, soybean trypsin inhibitor (SBTI), a control protein of a size similar to that of AHSP, had no effect. Therefore, AHSP specifically and directly inhibits the oxidation of Hb A by α -Hb. These findings are consistent with the ability of AHSP to inhibit ROS production by α -Hb and also can account for the presence of unstable Hb A in AHSP^{-/-} erythrocytes (Figures 1 and 4, above).

Loss of AHSP exacerbates β -thalassemia. If α -Hb is stabilized by AHSP in vivo, then loss of the latter could worsen β -thalassemia, a disorder in which erythroid cell damage is mediated by the accumulation of free α -Hb. To test this, we mated AHSP knockout mice with thalassemic ones. We used Hbb^{+/th-3} (β -globin^{+/th-3}) mice, which contain a targeted deletion of the adult β -globin genes *b1* (β major) and *b2* (β minor) (35). Heterozygous animals exhibit a β -thalassemia intermedia phenotype, while homozygotes die in utero from severe anemia. We mated β -globin^{+/th-3} heterozygotes to AHSP^{-/-} mice and then interbred the resulting F1 double-heterozygotes. Genetic analysis of the F1 intercrosses, shown in Figure 7 and Table 2, took into account the fact that AHSP and β -globin are physically linked on the same chromosome (indicated in Figure 7A). Fluorescent in situ hybridization using a bacterial artificial chromosome-derived probe localized AHSP to mouse chromosome 7, band F2-F3 (M.J. Weiss, unpublished data), while the β -globin locus resides on mouse chromosome 7, band E2-E3 ([http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?org=mouse&chr=7&maps=ugMm,model,mg&query=Hbb-b1\[SYM\]&cmd=focus&fill=40&size=40](http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?org=mouse&chr=7&maps=ugMm,model,mg&query=Hbb-b1[SYM]&cmd=focus&fill=40&size=40)). Parenthetically, AHSP and β -globin are on separate human chromosomes (16 and 11, respectively).

Among non- β -thalassemic progeny (Table 2), we observed an overall recombination rate of 0.25 between the β -globin and AHSP genes. Our breeding studies described above established that loss

**Table 2**Genotype distribution of live-born offspring from intercrosses of β -globin^{+/th-3}AHSP^{+/-} compound mutant mice

β -globin genotype	+/+			+/th-3		
AHSP genotype	+/+	+/-	-/-	+/+	+/-	-/-
Required meiotic recombinations	2	1	0	1	0 or 2	1
Observed offspring (<i>n</i> = 111)	3	13	22	21	46	6
Expected offspring ratio (θ = 0.25)	0.0625	0.3750	0.5625	0.1875	0.6250	0.1875
Expected offspring	2.4	14.2	21.4	13.7	45.6	13.7

The β -globin and AHSP genes are physically linked. The mutant alleles for each gene were on separate chromosomes (*trans* conformation) in double heterozygotes used for mating, according to the strategy described in Figure 7A. Line 3 indicates the number of meiotic recombination events required to generate the indicated compound genotypes. Since prior studies showed that AHSP loss does not affect the viability of β -globin^{+/+} mice, we used the observed number of recombination events in this group to calculate the recombination frequency (θ) between the two genes [(3 × 2) + 13/(38 × 2) = 0.25]. As predicted, the observed and expected genotype distributions (lines 4 and 6) are in agreement (Chi-squared = 0.27; *P* > 0.75). In contrast, among the β -globin^{+/th-3} offspring, the observed AHSP genotype distribution deviated significantly from the expected values (Chi-squared = 8.22; *P* < 0.025). In particular, the AHSP^{-/-} genotype was significantly underrepresented among live-born β -globin^{+/th-3} mice.

of AHSP does not affect viability when β -globin genes are wild-type. Given the number of meiotic recombination events required to generate each AHSP genotype, the distribution of observed offspring was in good agreement with expected values (Table 2). In marked contrast, the observed AHSP genotype distribution deviated significantly from the expected values in β -globin^{+/th-3} mice (Table 2). Specifically, the AHSP^{-/-} genotype was underrepresented, indicating that loss of AHSP impairs survival of embryos with thalassemia intermedia (β -globin^{+/th-3} genotype).

To investigate further the interactions between AHSP and β -thalassemia intermedia, we examined β -globin^{+/th-3}AHSP^{-/-} compound mutant embryos at embryonic day 16.5–17, a stage at which erythropoiesis is predominantly fetal liver derived and, therefore, is dependent upon the expression of adult-type β -globin genes. To favor the production of double-mutant embryos, we crossed β -globin^{+/th-3} mice that were either AHSP^{+/-} or AHSP^{-/-} with β -globin^{+/+} AHSP^{+/-} mice. All embryos with the β -globin^{+/th-3} genotype were pale (not shown) and anemic (Figure 7B). However, loss of AHSP reduced the mean hematocrit of thalassemic embryos from 27.5% to 22.6%. The double-mutant embryos showed greater variation in hematocrit, with values reaching as low as 16%. Moreover, the blood smears of thalassemic embryos lacking AHSP showed more inclusion bodies in mature erythrocytes and increased numbers of nucleated fetal liver-derived (definitive) erythrocytes, indicative of erythropoietic stress (Figure 7C). Therefore, it is likely that severe anemia impairs the viability of some β -globin^{+/th-3}AHSP^{-/-} embryos, causing this genotype to be under-represented among live-born pups (Figure 7A and Tables 1 and 2, above).

Analysis of late-stage β -globin^{+/th-3} embryos suggested that loss of AHSP exacerbates β -globin deficiency in definitive erythrocytes (Figure 7B, above). Consistent with this possibility, the AHSP^{-/-} animals with β -thalassemia intermedia that escaped embryonic death and developed into early adulthood were significantly more anemic than those with AHSP^{+/-} and AHSP^{-/-} genotypes (Table 3). In addition, AHSP^{-/-} thalassemic mice exhibited smaller erythrocytes (decreased MCV) and greater variations in erythrocyte size (RDW) and Hb content (HDW) (Table 3).

Erythrocytes of AHSP^{-/-} thalassemic mice showed more prominent inclusion bodies than did those of animals with thalasse-

mia alone (Figure 8A), similar to what was observed in the embryos (Figure 7B, above). This suggests that loss of AHSP might exacerbate globin precipitation in the setting of α -chain excess. Therefore, we compared membrane skeleton-associated globin chains in mice of various β -globin and AHSP mutant genotypes (Figure 8, B and C). As expected, predominantly α -globin precipitate was present in β -thalassemic erythrocytes. This is due to the unstable nature of free α -Hb combined with relative β -globin deficiency (5). Importantly, α -globin precipitates in β -thalassemic erythrocytes were significantly increased by deficiency in AHSP (Figure 8B, lanes 2 and 3 compared with lanes 4–6, and Figure 8C), further indicating a role for this protein as a molecular chaperone that neutralizes toxic free α -Hb.

Discussion

Erythrocyte precursors synthesize Hb A tetramer extensively, with minimal damage occurring from cytotoxic free heme and globin subunit precursors. Some of the mechanisms that balance Hb A synthesis and protect against toxicity of its components are recognized. For example, increased efficiency of β -globin translation compensates for higher levels of α -globin RNA transcribed from four active genes (36, 37). Evidence for molecular cross-talk to balance Hb A precursor production is also illustrated by two heme sensor proteins. Heme-regulated kinase inhibits globin translation when heme availability is limited (38). Bach I is a nuclear repressor that inhibits β -globin transcription at low heme concentrations (39). In addition, proteolytic pathways to degrade excess free α -Hb are present in erythroid cells (13–15). It is also to be expected that molecular chaperones exist to transiently stabilize Hb A synthetic intermediates prior to their assembly. α -Hb exists as a monomer, rendering it particularly unstable compared with β -Hb and Hb A, which exist mainly as tetramers under physiologic conditions. For this reason, a chaperone for α -Hb would be particularly advantageous; AHSP potentially serves this function.

AHSP is abundant in late-stage erythroid precursors, in which its expression kinetics parallel that of α -globin (40). AHSP specifically binds free α -Hb in a fashion that is not expected to interfere with Hb A assembly or function (16). Moreover, AHSP-bound α -Hb is resistant to denaturation and can still be incorporated into Hb A when

Table 3Effects of AHSP loss on erythrocyte indices of adult β -thalassemic (β -globin^{+/th-3}) mice

β -globin genotype	+/th-3	+/th-3	<i>P</i> value
AHSP genotype	+/+ (<i>n</i> = 6)	-/- (<i>n</i> = 6)	
Hb	11.7 ± 0.5	9.9 ± 0.8	0.002
Hct	35.3 ± 1.1	26.2 ± 3.8	< 0.001
Reticulocytes (%)	13.3 ± 4.8	15.3 ± 0.8	0.4
MCV	41.7 ± 1.7	38.7 ± 1.2	0.006
MCH	13.8 ± 0.4	14.7 ± 1.4	0.1
RDW	28.5 ± 1.9	32.4 ± 0.8	0.003
HDW	3.2 ± 0.1	4.0 ± 0.1	< 0.001

No significant differences were observed between AHSP^{+/-} and AHSP^{-/-} β -thalassemic mice.

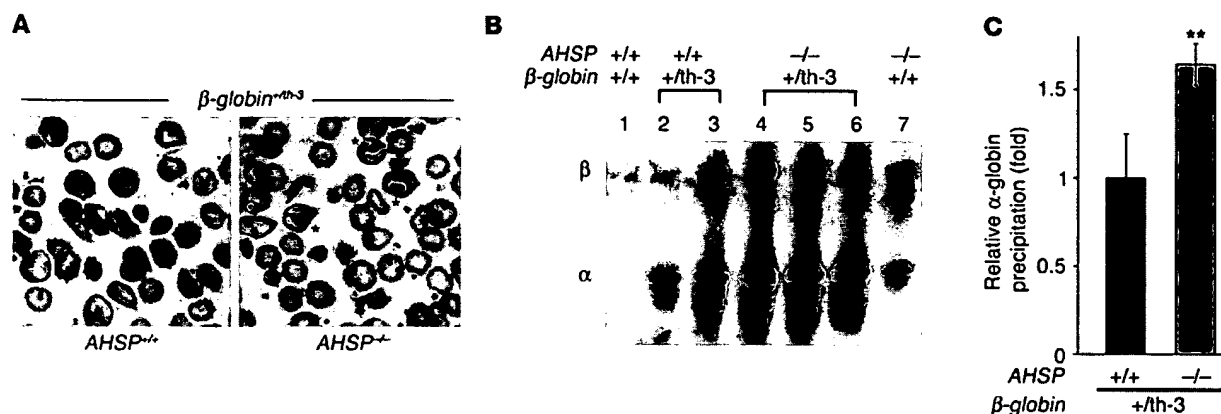


Figure 8

AHSP loss exacerbates α -globin precipitation in adult mice with β -thalassemia intermedia. (A) Blood smears of thalassemic mice (β -globin^{+/th-3}) with AHSP^{+/+} and AHSP^{-/-} genotypes. Eosinophilic inclusions (marked by asterisks) are more prominent in AHSP^{-/-} erythrocytes. Original magnification, $\times 100$. (B) TAU gel analysis of membrane skeleton-associated globin chain precipitates from mice presented in A. Each lane represents membrane skeletons prepared from the same number of erythrocytes. (C) Relative level of α -globin precipitation in thalassemic erythrocytes of different AHSP genotypes. Membrane skeleton-associated α -globin detected in TAU gels, as illustrated in B, was quantified using the NIH IMAGE software program (<http://rsb.info.nih.gov/ni-image/>). The average signal obtained from β -thalassemic animals with a wild-type AHSP genotype (black bar) was normalized to a value of 1. $n = 4$ animals of each genotype; ** $P < 0.01$. Heterozygous loss of AHSP had no significant effect on α -globin precipitation in β -thalassemic mice (not shown).

β -Hb is available (17). These observations are consistent with a model in which AHSP serves as a docking molecule to temporarily bind α -Hb, stabilize its structure, and render it biochemically inert prior to Hb A assembly. Our results here indicate that when AHSP is absent, α -Hb becomes unstable and generates ROS that damage α -Hb itself, Hb A, and other cellular constituents (Figure 9). AHSP could inhibit ROS production from α -Hb by reducing its inherent ability to participate in redox reactions and/or by inhibiting its denaturation and subsequent heme release (reviewed in refs. 7 and 41). In this regard, our recent data indicate that AHSP induces the conversion of α -Hb to a non-reactive form in which all six coordinate positions of heme-bound iron are liganded (42).

The toxicities of α -Hb are most thoroughly studied in the context of β -thalassemia, in which accumulation and precipitation of the free α -Hb subunit reduce the survival of circulating erythrocytes and also cause ineffective erythropoiesis associated with impaired viability and apoptosis of erythroid precursors. Similarly, AHSP^{-/-} mice exhibit shortened erythrocyte half-life with globin precipitates and ineffective erythropoiesis. Unpaired α -Hb is a potent oxidant and β -thalassemic erythrocytes exhibit increased ROS, depleted endogenous antioxidants, and pathological oxidation of cellular lipids and proteins (reviewed in ref. 7). AHSP^{-/-} erythrocytes also contain elevated ROS and signs of oxidative damage. Hence, β -thalassemia and AHSP deficiency share many pathological features. Our current findings indicate that the erythroid defects in AHSP^{-/-} mice stem from unstable α -Hb.

Damage to Hb A by ROS generated from unstable α -Hb probably accounts for the presence of α - and β -globin precipitates in equal proportions in the erythrocyte membrane of AHSP^{-/-} mice (Figure 5, above). In contrast, erythrocytes from mice and humans with β -thalassemia contain mainly α -globin precipitates (24, 43–46), despite high levels of ROS that might be expected to damage other expressed hemoglobins. This is due to excess free α -globin caused by β -globin deficiency (5). In our studies, erythrocyte β -globin precipitates were present in some mice with β -thalassemia intermedia alone, although

the relative levels of α -globin precipitates were always greater (Figure 8B and not shown). Concomitant AHSP deficiency exacerbated both α -globin precipitation and, to a lesser extent, β -globin precipitation in thalassemic mice. Therefore, while unstable α -Hb contributes greatly to the pathophysiology of both AHSP deficiency and β -thalassemia, impaired β -globin synthesis in the latter disorder results in a greater proportion of α -globin precipitation.

Our findings that AHSP protects against α -Hb-associated toxicities have potential implications for human disease. For example, defects in AHSP could be responsible for unexplained cases of Heinz body hemolytic anemia in humans. More importantly, variations in AHSP levels could modulate the severity of β -thalassemia in humans. In support of this, an abstract by Galanello et al. reported that reduced AHSP mRNA expression was associated

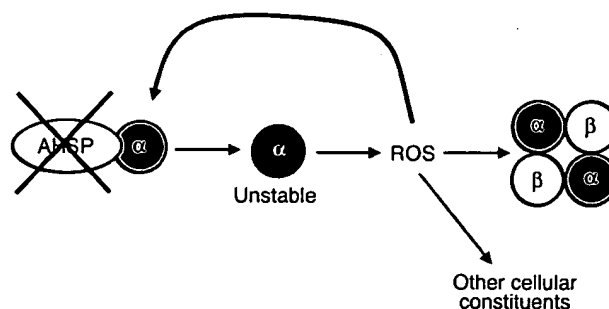


Figure 9

Consequences of AHSP loss in erythroid cells. Without AHSP, α -Hb becomes unstable, with an increased propensity to generate ROS. This leads to further damage and to the precipitation of α -Hb and Hb A, as well as to the oxidation of other cellular constituents. In addition, unstable α -Hb degrades, releasing toxic porphyrins, free iron, and denatured α -globin protein chains (not shown). The end result is premature loss of circulating erythroid cells and erythroid precursors.



with more severe β -thalassemia among related individuals with the same mutant β -globin alleles, although no mutations in the coding region or proximal promoter of the *AHSP* gene were detected (47). However, in another recent study, we mapped and sequenced the *AHSP* gene in 120 Thai patients with HbE- β -thalassemia of varying clinical phenotypes (48). We found no mutations or specific association between *AHSP* haplotypes and thalassemia severity, indicating that *AHSP* gene mutations are unlikely to influence thalassemia phenotypes in this population. Whether mutations in the *AHSP* gene or epigenetic events that alter its expression represent major modifiers of human β -thalassemia still remains an open question. To answer this, it will be important to study the *AHSP* gene, *AHSP* mRNA, and *AHSP* protein in additional β -thalassemic populations and pedigrees. Finally, given that many adverse effects of free α -Hb are relieved by *AHSP*, it is possible that the pursuit of drugs or peptides that mimic its activities will yield new pharmacological strategies for the treatment of β -thalassemia.

Methods

Animals. The generation and genotyping of *AHSP*-deficient mice was described previously (17). The β -thalassemic mice (*tb-3* allele) (35) were provided by Oliver Smithies (University of North Carolina, Chapel Hill, North Carolina, USA). Protocols were approved by the Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia (Philadelphia, Pennsylvania, USA). All animals were studied between 6 and 20 weeks of age.

Hematological analysis. Blood from adult mice was sampled retro-orbitally, anticoagulated with EDTA, and analyzed on a Bayer ADVIA 120 Hematology System. Hematocrit determination of embryos was performed using 2- μ l microcapillary tubes to pierce the carotid vessels and collect blood. Care was taken to prevent contamination of the samples with fluid surrounding the embryo. Three tubes were collected for each embryo. The first tube was filled halfway and discarded. Two additional tubes were filled and the hematocrits determined for each were compared. When values from duplicate samples differed by less than 5%, the average was calculated and used for our analysis (Figure 7B, below). When values differed by more than 5% in samples from the same embryo, we considered there to be a sampling error and data were discarded.

For determination of erythrocyte half-life, NHS-biotin was injected intraperitoneally at a dose of 150 mg/kg on two consecutive days (days -2 and -1 prior to analysis beginning at day 0). Five microliters of blood was drawn from the tail vein, incubated with FITC-streptavidin (BD Biosciences-Pharmingen), and analyzed on a FACSCalibur (BD) (18). For phenylhydrazine treatment, a dose of 63 mg/kg was injected intraperitoneally. Hematocrit and reticulocyte counts were analyzed for 9 days afterward.

Methylcellulose colony assays to quantify erythroid progenitors were performed as described (49). Splenocytes or bone marrow cells were plated in triplicate at a density of 10^5 per 30-mm dish. Cytokine combinations for specific progenitors were as follows: for CFUe, 2 U/ml Epo; for BFUe, 2 U/ml Epo and 50 ng/ml SCF; for myeloid, 1 ng/ml interleukin 3 (IL-3), 15 U/ml GM-CSF, and 100 U/ml M-CSF. Recombinant human Epo was purchased from Amgen. All other cytokines were mouse reagents and were purchased from R&D Systems.

Proteins. Recombinant *AHSP* and hemoglobins were purified as described (16).

Analysis of globin precipitates. Heinz body staining was performed as described previously (50). For globin chain analysis in membrane skeletons (51), 40 μ l of freshly drawn blood was lysed and washed extensively in 0.05% PBS. Membrane lipids were removed by extraction with 56 mM sodium borate (pH 8.0). Precipitated globins were dissolved in 8 M urea, 10% acetic acid, 10% β -mercaptoethanol, and 0.04% pyronin, were fractionated

on TAU gels, and stained with Coomassie brilliant blue. The fraction of sample loaded on the TAU gel was adjusted relative to the original hematocrit so that equal numbers of erythrocytes were represented in each lane.

Flow cytometry. Analysis to quantify erythroid maturation stages in spleen and bone marrow was performed as described previously (19). Single-cell suspensions were stained with PE-Ter119 and FITC-CD71 (BD Biosciences—Pharmingen). For exclusion of dead cells, 7-aminoactinomycin D (Sigma) was used. Studies were performed on a FACSCalibur System (BD). For detection of intracellular ROS, 10^7 peripheral blood cells were pre-loaded with DCFH (Sigma) and then were incubated with H_2O_2 as indicated in Figure 5A, and intracellular fluorescence intensity was detected by flow cytometry (52).

Detection of carbonyl-modified intracellular proteins. Protein was prepared from mouse erythrocytes and 20 μ g was analyzed with the Oxyblot Kit (Intergen) following the manufacturer's protocol.

Tissue collection and histopathology. Tissues for immunohistochemistry and histopathology were fixed in formalin, embedded in paraffin, and sectioned using standard procedures. Immunohistochemistry was performed using Ventana systems. The TUNEL assay was performed in accordance with the manufacturer's recommendations (Roche). A monoclonal antibody (LY-76; BD Biosciences-Pharmingen) against the Ter119 antigen was used to identify late-stage erythroid cells. Double labeling was accomplished with a combination of both techniques.

ROS production by α -Hb. A solution of 40 μ M oxygenated α -Hb with or without 40 μ M *AHSP* was pre-incubated on ice for 30 minutes. TMPD (Sigma) (34) was then added to a final concentration of 400 μ M and its oxidation was measured by continuous light absorbance monitoring at 610 nm. The rate of oxidation was calculated based on the slope of the initial 10 minutes of the reaction. Heme loss was measured by the decrease of light absorbance in the Soret range (412 nm).

Hb A oxidation induced by its subunits. Oxygenated α -Hb (5 μ M) was incubated on ice for 30 minutes with 5 μ M *AHSP* [or SBTI (Sigma) as negative control] to form the α -Hb-*AHSP* complex. This complex was added to a mixture of 40 μ M oxygenated Hb A and 1 mM reduced glutathione (28). The reaction was incubated at 37°C for 30 minutes and the fraction of Fe(III) Hb was calculated by linear regression to extinction coefficient spectra over the wavelength range of 500–700 nm (53).

Statistical methods. The unpaired 2-sample Student's *t* test was used for statistical analysis in Figures 1–8 and in Tables 1 and 3. A *P* value of less than 0.05 was considered to be significant. For genetic linkage analysis (Figure 7A and Table 2) we used the chi-squared test.

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